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Cell Wall-Associated Proteases of *Streptococcus cremoris* Wg2

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Two components of the proteolytic system, proteins A and B (J. Hugenholtz, F. Exterkate, and W. N. Konings, Appl. Environ. Microbiol. 48:1105-1110, 1984), have been studied in *Streptococcus cremoris* Wg2 by immunological methods. The components could not be separated by standard chromatography techniques because both proteins had almost identical molecular weights (about 140,000) and isoelectric points (pH 4.5). Specific antibodies were raised against proteins A and B by excision of the different immunoprecipitates from crossed immunoelectrophoresis gels. With these antibodies, protein A or B was removed from solutions containing both proteins. The purified proteins A and B possessed proteolytic activity and were inhibited by the serine protease inhibitor phenylmethylsulfonyl fluoride. Each of these proteins accounted for approximately 50% of the total proteolytic activity isolated from *S. cremoris* Wg2. The specific antibodies against the proteases were also used for immuno-gold labeling studies. The proteases were clearly seen to be located at the outside of the cell wall. The proteases had the same location when the genetic information coding for the proteases was cloned in *Streptococcus lactis* and *Bacillus subtilis*.

Lactic acid bacteria have two main functions as starter bacteria in the manufacturing of cheese: (i) the acidification of milk and (ii) the development of flavor during the ripening. In both processes, the proteolytic activity of these bacteria plays a central role. For growth and subsequent acidification in milk, the bacteria need the proteases for their supply of essential amino acids by the hydrolysis of milk protein. The same proteases continue to be active long after the bacteria have ceased to grow, and during this time of ripening, the casein in the cheese is slowly hydrolyzed by the bacterial proteases. In this process, amino acids and peptides are produced which add to the flavor of cheese.

Because of the importance of the proteases in cheese production, much attention has been focused on these enzymes in the study of starter bacteria in recent years. The collected data on the proteases of group N streptococci are mostly confusing. The molecular weights of the proteases have been found to vary from 130,000 to 180,000 (11, 13, 21), although lower molecular weights have been reported (5). The number of different proteases in one strain has been claimed to be 1 (11, 21) to 4 (3, 13), and the localization of the proteases in the cell has been found to be either extracellular (8), cell wall associated (9-11, 13, 20, 28), membrane bound (25), or cytoplasmic (1, 2, 5, 21). With respect to one characteristic feature, most investigators seem to agree; Ca^{2+} ions are important for the activity of the proteases (9, 11, 13, 20, 21), although in one case, Mn^{2+} and Co^{2+} ions were mentioned instead of Ca^{2+} as cofactors (5).

In a recent paper (13), we contributed to this confusion by immunological analysis of the proteolytic systems of several *Streptococcus cremoris* strains that were known to have different proteolytic activities (8). All the different proteolytic systems consisted of two or more components, one (protein A) of which was present in all the strains tested. This protein was identified as a protease. The proteolytic nature of the other proteins could not be determined. The different components could not be correlated with the differences in proteolytic activity, as reported by Exterkate (8).

In this work, we have attempted to resolve some of these problems by studying the proteolytic system of one strain, *S. cremoris* Wg2, in more detail and have analyzed the characteristics of the two components of the proteolytic system in this strain, protein A and protein B. Furthermore, we have used immuno-gold labeling to determine the exact localization of the proteases in the cell.

MATERIALS AND METHODS

Bacterial strains. *S. cremoris* Wg2 and *S. cremoris* E8 were obtained from The Netherlands Institute for Dairy Research (Ede, The Netherlands). *S. lactis* MG1363 (pGKV500) and *Bacillus subtilis* PSL1 (pGKV500) (16) were obtained from the Department of Molecular Genetics, University of Groningen. The strains were routinely stored in 10% skim milk at -20°C .

Protease isolation. *S. cremoris* Wg2 was grown in 5 liters of MRS medium (4) with 10 mM CaCl_2 and 2% lactose. The pH of the culture was maintained at 6.3 by automatic titration with 2 N NaOH. The culture was harvested when all the lactose in the medium was consumed and the cells had stopped growing at a cell density at A_{660} of about 4.0.

S. cremoris E8 was grown in 5 liters of synthetic RFP medium, which was prepared as described previously with 10 mM CaCl_2 , 2% lactose, and 0.8% sodium caseinate instead of the usual amino acid mixture (13, 14). The culture was grown and harvested in the same way as described above.

The proteases were isolated from the cells as described previously (13, 20) by incubating the washed cells for 30 min at 30°C in Ca^{2+} -free phosphate buffer. This incubation was repeated three times to increase the amount of proteases isolated.

Partial purification of the proteases. (i) **DEAE-Sephacel chromatography.** The crude isolated protease solution (about 1 liter) was eluted over a DEAE-Sephacel column (length, 25 cm; surface area, 6.5 cm) with a NaCl gradient of 0.1 to 0.3 M in 20 mM Tris hydrochloride (pH 7.8). The proteolytic activity was eluted in an NaCl concentration of 0.13 M. The fractions containing proteolytic activity were pooled, concentrated 20- to 50-fold by ultrafiltration with a Minicon B15

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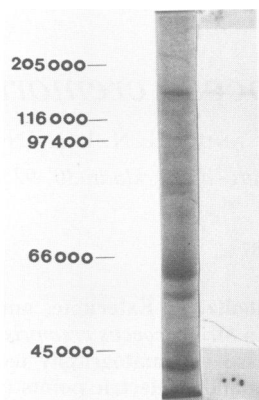


FIG. 1. SDS-PAA gel electrophoresis of the partially purified proteases of *S. cremoris* Wg2. The right side of the figure shows the immunoblotting of the proteases with specific antibodies against the proteases of *S. cremoris* Wg2.

concentrator (cutoff, 15,000 M_w ; Amicon Corp., Danvers, Mass.) and used for analysis by gel electrophoresis or for further purification.

(ii) **Sephacryl S-300 chromatography.** The pooled and concentrated fractions of the DEAE-Sephacel eluate were dialyzed three times for 8 h each time against 50 mM potassium phosphate (pH 7.0) to remove the salt. Subsequently, they were eluted in a Sephacryl S-300 column (length, 70 cm; diameter, 0.9 cm) with 20 mM Tris hydrochloride (pH 7.8). The fractions containing proteolytic activity were pooled and used for gel electrophoresis. Purified protease of *S. cremoris* HP was supplied by The Netherlands Institute for Dairy Research as freeze-dried samples giving one protein band when applied on isoelectric focusing (IEF) gels (F. A. Exterkate, personal communication).

SDS-PAA gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide (SDS-PAA) gel electrophoresis was performed as described previously (17). In most cases, 7.5% PAA was used. The protease samples were mixed 1:1 with sample buffer (0.18 M Tris hydrochloride [pH 6.8], 12.9% SDS, 8.6% glycerol, 0.07% bromophenol blue) and incubated at 100°C for a maximum of 5 min. Longer incubation resulted in disintegration of the proteases (data not shown). Silver staining of the gels was performed according to Wray et al. (30). For determination of the molecular size, the following reference proteins were used: albumin (45,000 and 66,000 daltons [Da]), phosphorylase *b* (97,400 Da), β -galactosidase (116,000 Da), and myosine (205,000 Da).

CIE. Crossed immunoelectrophoresis (CIE) was carried out as described previously (7, 28).

IEF. IEF on slab gels was performed on ready-to-use Servalyt Precotes (pH 3 to 10; Serva, Heidelberg, Federal Republic of Germany). For the determination of the isoelectric point, the following references were used: ferritin (pI 4.4), ribonuclease (pI 9.45), and cytochrome *c* (pI 10.65).

For two-dimensional IEF-CIE, the IEF in the first dimension was performed with cylindrical gels as described previously (12). For the CIE in the second dimension, the cylindrical gels were transferred to glass plates (5 by 15 cm) and embedded in 1% agarose containing antibodies.

Immunoblotting. SDS-PAA gels were blotted on nitrocellulose sheets by the Western blotting technique, and the proteases were stained with peroxidase-conjugated specific antibodies as described by Towbin et al. (27). For the

immunological detection, the antibodies against the proteases were diluted 50-fold, and conjugation with peroxidase was achieved by using 2,000-fold-diluted peroxidase-conjugated swine anti-rabbit immunoglobulin G (DAKO Immunoglobulins, Denmark).

Isolation of specific antibodies. Specific antibodies against proteins A and B were obtained by excision of the appropriate immunoprecipitates from CIE gels. Ten of these gel slices were homogenized in 2.5 ml of 0.9% NaCl and injected into rabbits as described previously (7, 29). The antibodies were isolated from the rabbit serum as described previously (7, 29).

Immunoabsorption. To 100 μ l of the partially purified proteases (100 μ g of protein per ml) were added 50 to 350 μ l of antibodies (5 mg of protein per ml) against protein A or B and 300 to 0 μ l of 20 mM sodium barbiturate buffer (pH 8.6) to adjust the volume of the samples to 450 μ l. These mixtures were incubated at 25°C for 30 min, and precipitates were removed by centrifugation at $48,000 \times g$ for 90 min. The supernatants were used for CIE experiments (10 μ l) or measurement of the proteolytic activity (100 μ l).

Immuno-gold labeling. The labeling of the proteases in the cells of *S. cremoris* with gold particles was performed as described previously (6) by treating cross sections of Lowicryll-embedded and fixed cells with specific antibodies against proteins A and B, followed by incubation with protein A-gold particles (12 nm).

Protease activity measurement. Proteolytic activity was determined as described previously (24) by using 1% fluorescamine-labeled casein as the substrate.

Protein determination. Protein concentrations were determined by the method of Lowry et al. (19) with bovine serum albumin as the standard.

RESULTS

Purification of the protease of *S. cremoris* Wg2. The proteolytic system of *S. cremoris* Wg2 has been shown to contain two proteins that are immunologically unrelated. This was demonstrated on CIE gels in which two major precipitates could be distinguished representing proteins A and B (13). Both proteins appeared to be part of the proteolytic system since they were absent in proteolytically negative variants of *S. cremoris* Wg2. Protein A could be identified as a protease by a zymogram technique, but attempts to demonstrate such activity for protein B were unsuccessful.

We have attempted to separate both proteins so that they could be characterized individually. Gel filtration (Sephacryl S-300) and ion-exchange chromatography (DEAE-Sephacel) were not successful in separating the proteins, as could be seen in the CIE pattern of the purified proteolytic system showing the precipitates of both proteins A and B (see references 13 and Fig. 3A). Moreover, repeated elution over DEAE-Sephacel and Sephacryl S-300 did not result in a separation of the proteins. They apparently had identical molecular weights and identical isoelectric points. This was indeed demonstrated by applying samples of the partially purified proteolytic system on SDS-PAA gels (Fig. 1) and on IEF gels (Fig. 2). In both cases, several protein bands were seen on the gels, the most prominent one representing a molecular weight of 140,000 and an isoelectric point of pH 4.5. The major band on both gels was identified as consisting of proteins A and B by (i) immunoblotting of the SDS-PAA gels and (ii) CIE in the second dimension of the IEF gels. In both cases, the specific antibodies against proteins A and B

were used (see below). The immunoblotting resulted in the staining of only the 140,000-Da protein band (Fig. 1), and the IEF-CIE gels showed several precipitates originating from the protein(s) with an isoelectric point of pH 4.5 (Fig. 2). If the protease samples were handled for longer periods at above-freezing temperatures before application on the gels, many more protein precipitation bands with lower molecular weights were observed (data not shown). This was presumably caused by autolysis of the protease(s). The purified protease of *S. cremoris* HP, which produced one protein band on IEF gels (F. A. Exterkate, personal communication) was also found to contain proteins A and B in CIE experiments (13; data not shown).

Separation of the two proteins could only be accomplished when the proteolytic system of *S. cremoris* was first treated with the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) followed by elution in DEAE-Sephacel. Under these conditions, protein A was released from the ion-exchange material at a lower NaCl concentration than was protein B (data not shown). Presumably, PMSF did not bind to both proteins to the same extent. After this separation, it was not possible to characterize the activity of the proteins, since treatment with PMSF had inactivated the proteases completely and irreversibly.

Separation of proteins A and B by immunoabsorption. To separate proteins A and B in an enzymatically active state, we concentrated on the only difference known to exist between these proteins: their antigenic reaction. In a previous paper, the different proteolytic systems of *S. cremoris* were characterized immunologically (13). In all strains tested, protein A was found. By using antibodies against the proteolytic system of another *S. cremoris* strain which contained protein A but not B, it should be possible to remove selectively this protein A from the solution by immunoprecipitation. The proteolytic system of *S. cremoris* Wg2 was incubated with increasing concentrations of antibodies against the proteolytic system of *S. cremoris* E8 (containing proteins A and C). The resulting precipitates were removed by centrifugation, and the supernatants were analyzed for protein content and proteolytic activity. Protein A, protein B, and the proteolytic activity were found to decrease simultaneously. Apparently, the antibodies against the proteolytic system of *S. cremoris* E8 reacted just as well with protein A as with protein B. CIE studies of the

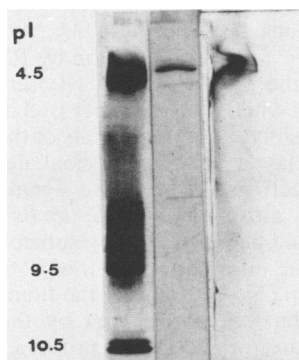


FIG. 2. IEF of the partially purified proteases of *S. cremoris* Wg2. On the left side, a slab gel is shown with standard proteins with isoelectric points of pI 4.5, 9.5, and 10.5. On the right side, a two-dimensional IEF-CIE gel is shown by using a cylindrical IEF gel with the same pH range (pH 3.5 to 10) as that used for the slab gel.

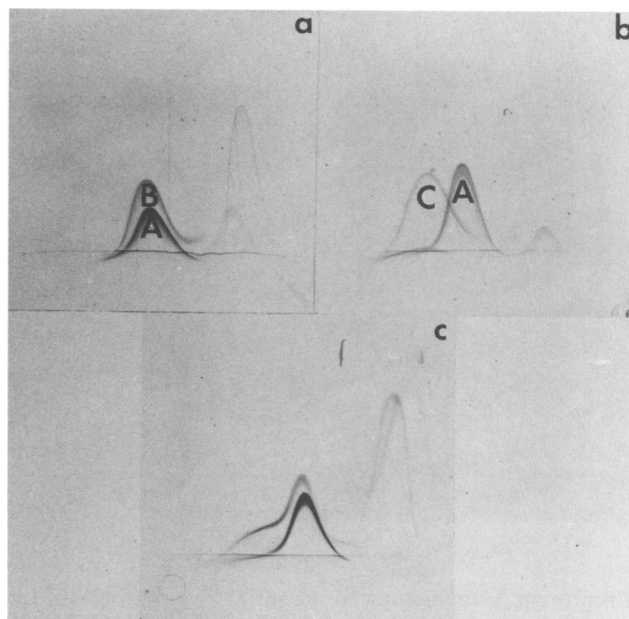


FIG. 3. CIE of the *S. cremoris* Wg2 proteolytic system (a), of the *S. cremoris* E8 proteolytic system (b), and of both together on one gel (c) by using antibodies against the proteolytic system of *S. cremoris* Wg2. Panels a, b, and c represent the proteolytic proteins A, B, and C, respectively.

proteolytic systems of *S. cremoris* Wg2 and *S. cremoris* E8 revealed that proteins B and C were immunologically identical (Fig. 3). This method could therefore not be used for the separation of protein A from protein B.

To achieve successful separation, specific antibodies against only protein A and against only protein B were subsequently used. These antibodies were obtained by excision of the precipitates of either protein A or protein B from CIE gels as described in Materials and Methods. The gel material was used as an antigen for raising new antibodies in rabbits. With these specific antibodies, it was possible to specifically remove by precipitation either protein A or protein B from solutions containing both proteins and to obtain preparations containing exclusively protein A or exclusively protein B. The immunoabsorption technique is illustrated in Fig. 4A, where protein A was seen to disappear from the CIE gels when increasing amounts of antibody against protein A were added to the samples before application on the CIE gels. In the same way, protein B could be removed by immunoabsorption (Fig. 4B). After removal of protein A, as indicated by CIE, the protease activity was decreased by 60%, and after removal of protein B, activity was decreased by 55%. These results indicate that *S. cremoris* Wg2 produced proteins A and B with about equal protease activities.

Proteolytic activity of proteins A and B. The successful separation of proteins A and B made it possible to determine the proteolytic activity of each individual protein. When protein A was removed from the samples by precipitation with specific antibodies, the proteolytic activity of the remaining protein solution gradually decreased to about 45% of the original activity. The CIE patterns of these treated samples are shown in Fig. 4A. When protein B was removed in the same way (Fig. 4B), about 55% of the proteolytic activity remained. These results show clearly that *S. cremoris* Wg2 contains two proteases, proteins A and B,

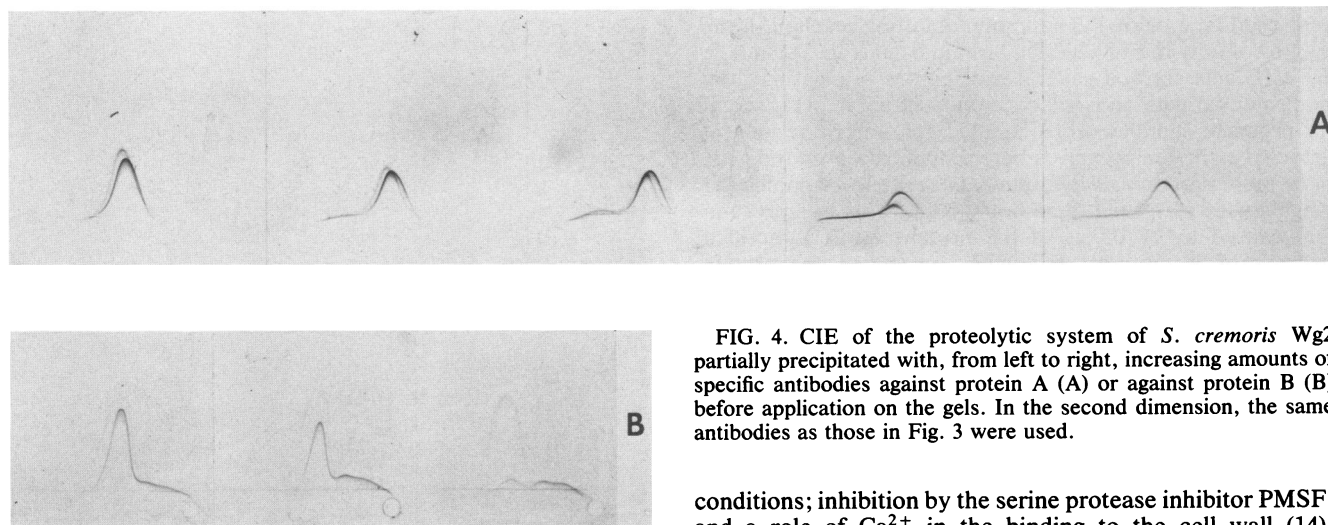


FIG. 4. CIE of the proteolytic system of *S. cremoris* Wg2 partially precipitated with, from left to right, increasing amounts of specific antibodies against protein A (A) or against protein B (B) before application on the gels. In the second dimension, the same antibodies as those in Fig. 3 were used.

with protein A accounting for about 55% and protein B for the remainder of the total isolated proteolytic activity.

Localization of the proteases by immuno-gold labeling. The specific antibodies against the proteases of *S. cremoris* Wg2 were also used to examine the localization of the proteases by immuno-gold labeling. Colloidal gold particles were attached to immunoglobulin molecules as described in Materials and Methods, making the antibodies visible under the electron microscope. In this manner, it is possible to determine the exact location of specific antigens (proteins) in the cell. The bacterial cells were fixed as quickly as possible to avoid disturbances of the in vivo structure of the cells. Slices of the embedded cultures were made and subsequently incubated with the gold-labeled antibodies. In Fig. 5A the immuno-gold labeling of the proteases of *S. cremoris* Wg2 is shown. Gold particles could only be found in the periphery of the cells. This indicated that the proteases were associated with either the cell wall or the cytoplasmic membrane. In slices containing cell wall material, gold particles were seen, also located on the outside of the cell material (data not shown). This clearly indicated that the proteases in *S. cremoris* Wg2 are located on the outside of the cell wall.

Immuno-gold labeling of the proteases in *S. lactis* and *B. subtilis*. The genetic information for the proteases of *S. cremoris* Wg2 was cloned into plasmid pGKV500 and was found to be expressed in *S. lactis* and *B. subtilis* (16). To determine the localization of the proteases in these heterologous organisms, immuno-gold labeling experiments were performed with antibodies against proteins A and B, as described above. The results for *S. lactis* MG1363 (pGKV500) (Fig. 5B) and *B. subtilis* PSL1(pGKV500) (Fig. 5C) were very similar. In both cases, the gold particles were found almost exclusively at the outside of the cell, and gold was found in the cell wall material. The localization of the proteases of *S. cremoris* Wg2 apparently was not changed after their genetic information was cloned and expressed in the other bacteria.

DISCUSSION

The following physicochemical characteristics of the *S. cremoris* Wg2 proteases are consistent with earlier reports on proteases of group N streptococci: M_w , 140,000; the absence of subunits after electrophoresis under denaturing

conditions; inhibition by the serine protease inhibitor PMSF; and a role of Ca^{2+} in the binding to the cell wall (14). Furthermore, the proteases were found to have an isoelectric point of pH 4.5, which is the same as that reported for the proteases of *S. cremoris* AC1 (11) and *S. cremoris* HP (F. A. Exterkate, personal communication).

Large discrepancies are found in the literature concerning the localization of the proteases in the cells of group N streptococci. This is probably due to the indirect, and therefore unreliable, way of determining the exact localization of these enzymes. These determinations were based mostly on fractionations of the cell with (8, 15, 18) or without (21, 26) lysozyme treatment by using more or less gentle methods for disruption of the cells or spheroplasts (25). In some reports, proteases were merely isolated from cell extracts and designated as intracellular (21). Obviously, the localization of the proteases has never been determined in a direct way. We have visualized the localization of the proteases of *S. cremoris* Wg2 by immuno-gold labeling (Fig. 5A). It can be seen clearly that the enzymes are located on the outer side of the cell wall. It seems functional that the proteases needed for the breakdown of casein are located on the outside of the cells. Casein molecules are large and are mainly present in an insoluble complex with Ca^{2+} ions. It is hard to imagine how intracellular proteases can play a role in the hydrolysis of casein. Most bacteria, of course, contain intracellular proteases for the turnover of the intracellular proteins. Such a protease was characterized for *S. diacetylactis* CNRZ 267, and its properties were quite different from the ones described here: M_w , 49,500; stimulation by Mn^{2+} and Co^{2+} ; and no inhibition by PMSF (5).

Interestingly, the proteases of *S. cremoris* Wg2 have the same localization when they are expressed in *S. lactis* and *B. subtilis* (16). Possibly, targeting signals of the proteases of *S. cremoris* Wg2 play a role in the final destination of the enzymes in the cell, and if so, these signals are recognized not only by the closely related *S. lactis* but also by *B. subtilis*. This could be elucidated by subcloning and expression of the genetic information for the proteases.

Differences can also be found in the literature concerning the substrates that are hydrolyzed by the proteases and which products are formed in this process. The protease(s) of *S. cremoris* AM1 is reported to specifically hydrolyze α -casein (23). This was also the case for the protease of *S. lactis* 3 (25). However, β -casein seemed to be the preferred substrate for whole cells of several other *S. cremoris* strains (8, 23). These strains showed a limited capacity to hydrolyze κ -casein, but no α -casein breakdown was observed. The

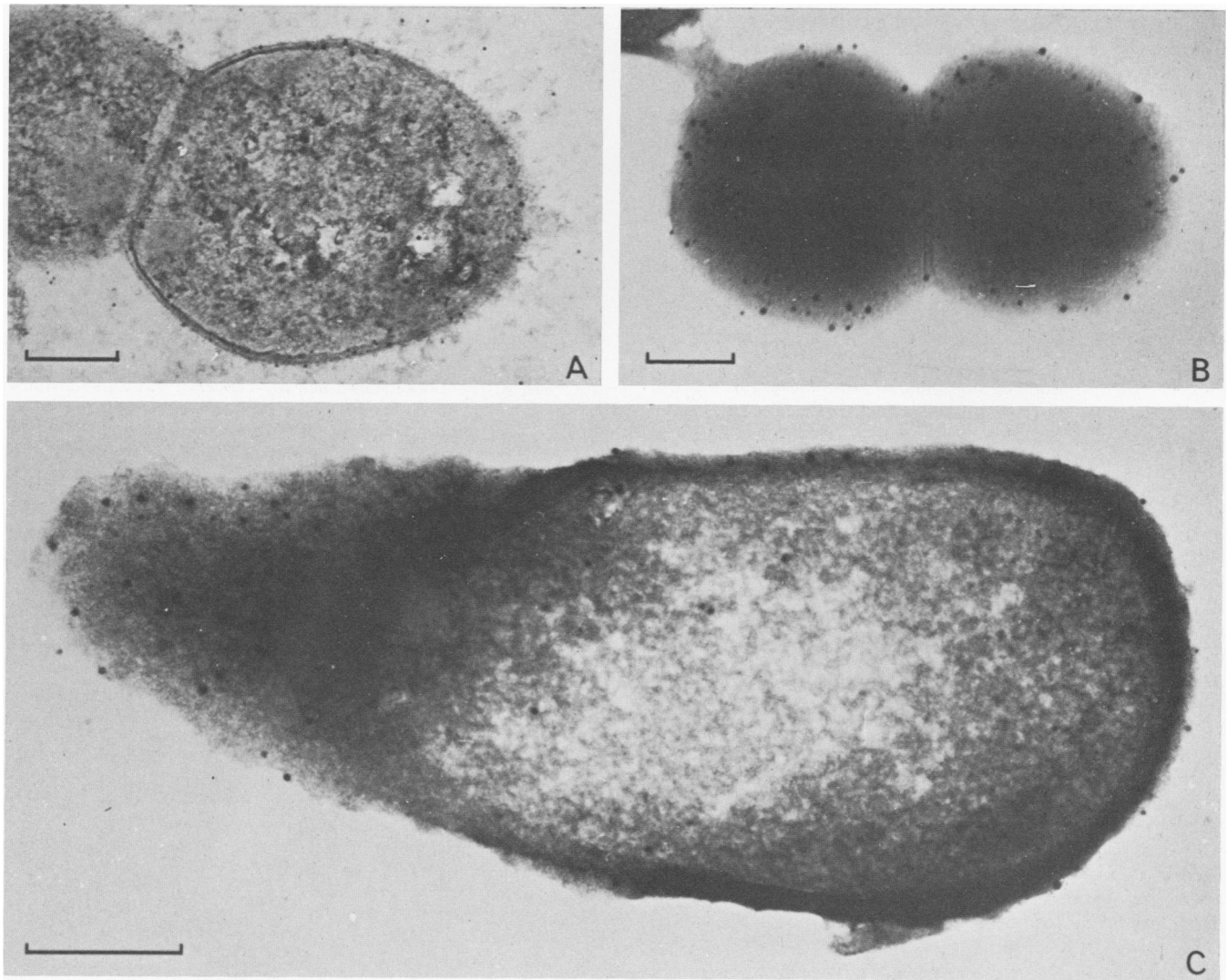


FIG. 5. Immuno-gold labeling of the proteases of *S. cremoris* Wg2 in cell slices of *S. cremoris* Wg2 (A), *S. lactis* MG1363(pGKV500) (B), and *B. subtilis* PSL1(pGKV500) (C). Specific antibodies against proteins A and B were used. Bar, 1 μ m.

purified proteases of *S. cremoris* AC1 (11) and HP (10) are able to degrade only β -casein and mainly β -casein, respectively. For *S. cremoris* Wg2, β -casein also seemed to be the only substrate for the proteases. When a synthetic medium with purified β -casein as the only nitrogen source was used, growth rates of the cells were identical to those on medium with total milk protein, and the same proteases were isolated under these conditions (data not shown). The proteases of *S. cremoris* H61 (21, 28) and *S. diacetylactis* CNRZ 267 (5) are reported to use all three caseins as substrates for proteolysis. The protease of the latter strain, however, was isolated from the cytoplasm by using cell extracts and was found to have different properties, including different substrate specificity.

Much work still has to be done to identify the products formed during casein hydrolysis by streptococcal proteases. Different strains of *S. cremoris* are reported to produce various amounts of (bitter) flavor after incubation with casein (8, 23). This difference in flavor has been attributed to different proteolytic activities of the strains (8). In our previous work (13), we observed differences in the (protein) composition of the proteolytic systems in the strains. One protease (protein A), however, was found in all the strains

tested. The differences in proteolytic activity between the strains is apparently determined by the other components of the proteolytic system. For *S. cremoris* Wg2, this paper shows that a second component, protein B, also has proteolytic activity. Protein C in *S. cremoris* E8, which shared antigenic determinants with protein B but showed a difference in electrophoretic mobility (Fig. 3), is presumably also a protease. *S. cremoris* Wg2 (and *S. cremoris* HP) is a bitter-producing strain, and *S. cremoris* E8 is not, which might suggest that protein B is responsible for bitter production in cheese.

The present data on the number of different proteases are in apparent contradiction with the observations of Geis et al. (11) and Ohmiya and Sato (21), who found only one protease in their strains. Their conclusions, however, are based on SDS-PAA gel electrophoresis studies, which are less sensitive than is the immunological approach used in this investigation. The two proteases of *S. cremoris* Wg2 were copurified by using gel filtration and ion-exchange chromatography and appeared as one protein band on SDS-PAA and IEF gels. When not applying an immunological method, one could easily conclude that there is only one protease in

these cells. The occurrence of more than one protease in streptococci was also reported by Cliffe and Law (3) and Akuzawa et al. (1, 2) in *S. lactis*. Exterkate described several proteolytic activities in whole cells of *S. cremoris* (8), but it is not known whether these are due to separate proteins. The possibility remains that the occurrence of more than one protease in the streptococci is a result of autolysis of one, original protease. To explain the data described here, the product of autolysis should not be much smaller than the original protease, and somehow, the antigenic determinants of the product would have to be different from the original protease.

The genetic information coding for the proteases in *S. cremoris* Wg2 was identified a few years ago. It is located on an unstable 17.5-megadalton plasmid (22). Recently, a 4.3-megadalton fragment of this plasmid was cloned in a protease-deficient *S. lactis* strain and in *B. subtilis*. Proteins A and B were expressed in both organisms, as could be seen in CIE experiments and by the regained ability of the proteolytically negative *S. lactis* to grow in milk (16). The genetic information of protein A, however, seemed to be incomplete, resulting in expression of a slightly aberrant, although still active, protein A (16). This may be in agreement with the size of the DNA fragment (4.3 megadaltons), which is not sufficiently large to code for the production of two proteins of 140,000 Da. The DNA sequence of this fragment is currently being determined, which will possibly give further insight into this matter. Proteins A and B can now be separated and purified on a large scale by using the specific antibodies in affinity chromatography. With the purified enzymes, it will be possible to compare both proteases with respect to activity and product formation.

It can be concluded that *S. cremoris* Wg2 contains at least two proteases, proteins A and B, both of which are located on the outside of the cell wall. Both proteases can use only β -casein as a substrate, both have a molecular weight of about 140,000 and an isoelectric point of pH 4.5, and both are inhibited by the serine protease inhibitor PMSF. Protein B is possibly responsible for the production of bitter peptides in this strain.

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